BOTULINUM TOXOIDS

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Botulism in human beings is a rare disease. As a result, there exists no need for the immunization of large numbers of persons against it. Immunization for laboratory workers engaged in studies and research on botulinum toxins would, however, be useful. The disease occurs worldwide,[1] though its geographic distribution is irregular.[2]

In some areas it has been a serious problem in domestic animals and in water fowl. Its prevalence in Western Australia among sheep and cattle and in South Africa among cattle fed on toxic carrion and among domestic mink fed on improperly handled mammalian or fish feed has warranted immunization of these animals against this disease.

Much has been published on the preparation of botulinum toxoids since the first reports by Weinberg and Goy in 1924,[3] and the antigenicity of botulinum toxoids prepared in various ways was demonstrated in experimental animals by a number of early investigators.[4–9] The experimental immunization of man with toxoid was reported in 1934 and 1936 by Velikanov.[10, 11] Melnik and Starobinetz[12] in 1936 showed that an alum-precipitated toxoid produced antitoxin in guinea pigs. Preparation of an effective type C toxoid for the control of botulism in sheep and cattle in Australia was described by Bennetts and Hall in 1938.[13]

Mason[14] and Sterne[15] and associates in South Africa demonstrated that mass immunization of cattle with combined types C and D formal toxoids effectively and economically reduced cattle losses resulting from botulism. Research was intensified during World War II, and methods for the preparation of alum-precipitated types A and B toxoids of high antigenic value in experimental animals and man were developed by Nigg and associates[16, 17] in the United States and by Rice et al.[18–20] in Canada. Prevot and Brygoo[21] produced type C antitoxin in man by immunization with toxoid prepared from toxin extracted from bacterial cells. Methods for preparation of type C toxoid for control of botulism in domestic mink[22–32] and game birds[33–36] have also been described. Barron and Reed[37] presented a systematic study of methods for preparation of crude alum-precipitated type E toxoid. Crude type E and type F toxoids have been prepared for the immunization of animals and the preparation of antitoxins.[38–50]

Although the bivalent type AB toxoid developed and produced at Fort Detrick during World War II produced satisfactory immunity in man,[51] as measured by serum antitoxin concentration, undesirable local and systemic reactions to injections of the relatively crude antigens were encountered. Subsequently, procedures for preparation of a purified polyvalent toxoid of improved clinical acceptability were investigated in our laboratories. The purpose of this review is to discuss the development of the purified botulinum toxoids and their evaluation in man and laboratory animals. The information presented represents a summation of published investigations carried out in our laboratories by Duff,[52–54] Wright,[55] Gordon,[56] Fiock,[57–59] Cardella,[60–63] and our associates. A brief outline of the methods employed for the preparation of the purified toxins and toxoids is presented first. Following this, data on the assessment in man of univalent, bivalent, and pentavalent preparations are presented. The concluding section of this paper presents studies on the efficacy of

toxoids, based on antitoxin titer and resistance developed by actively immunized animals to toxins administered by various routes.

PREPARATION OF PURIFIED TOXINS AND TOXOIDS

Production of high-potency types A, B, C, D, and E toxins was accomplished by growth of highly toxigenic strains of *Clostridium botulinum* in culture media that were composed of commercially available constituents.[52, 53, 56, 57, 60, 61] Production methods could be applied readily on a scale suitable for routine production of a biological product. Toxicity of type E toxin was increased 50- to 100-fold by treatment with trypsin.[54] The activation procedure allowed attainment of culture toxicities of the same order of magnitude as those obtained with types A, B, C, and D. These results provide the explanation for the low apparent toxicity of type E cultures — an observation that had been difficult to understand in view of the high mortality associated with outbreaks of type E botulism. The five toxins were purified by chemical methods that included an initial precipitation, extraction with calcium chloride, one or two reprecipitations, and subsequent resolution into an appropriate buffer system.[52, 53, 56, 57, 60, 61] Specific activity of the purified toxins ranged from 40 x 10^6 mouse intraperitoneal (MIP) LD_{50} per milligram N with the type C to approximately 500 x 10^6 MIP LD_{50} per milligram N with the type D toxin. Ultracentrifugal analysis showed that types A and B sedimented as a single boundary. With types C, D, and E, the major sedimentary boundary represented 80 to 90 percent of the protein concentration. The toxins were sterilized by filtration and converted to toxoids by incubation in the presence of formalin. Detoxification reactions deviated markedly from first-order behavior.[55] Toxin preparations were usually detoxified in 15 to 25 days in the presence of 0.6 percent formalin at 35° to 37°C and pH 5.5 to 6.5. Although the toxins were initially more toxic for mice than for guinea pigs, detoxification for mice occurred more rapidly than detoxification for guinea pigs. Fluid toxoids were readily adsorbed on aluminum phosphate, prepared as described by Holt[64] for diphtheria toxoids. Adsorbed toxoids were prepared so that 1 milliliter of final toxoid contained 7 milligrams of adjuvant. Thimerosal in a concentration of 0.01 percent was used as preservative. Gentle shaking for 18 to 24 hours was sufficient for adsorption of the toxoids. Safety testing included tests for the presence of toxin and standard sterility tests.

Procedures developed in our laboratories at Fort Detrick were subsequently adapted for large-scale production by Parke, Davis & Co. under the direction of Dr. Henry B. Devlin. Univalent and polyvalent preparations of adsorbed toxoid were produced for experimental use. Clinical trials with the toxoids have been completed. Toxoids were administered in either 0.25 or 0.5 milliliter amounts by deep subcutaneous inoculation in the deltoid region. The purified toxoids were well tolerated and were considered satisfactory clinically. Considerably less than 1 percent of those immunized showed a mild local reaction in the form of a transient nodule of varying size that persisted for approximately 2 weeks.

Individual antigenic responses to botulinum toxoids were determined by serum neutralization titrations in mice, with the univalent Porton (British) antitoxins* as primary reference standards. These Porton antitoxins were established as International Standards at the 15th Session of the Expert Committee on Biological Standardization, World Health Organization, Geneva, December 1962.[65] Purified toxins diluted with

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two parts glycerine and stored at minus 20°C were standardized against the reference antitoxins.

The units of antitoxin used for the standardization of the glycerinated toxins are presented in Table 1. The neutralizing capacities of the units of the five types of antitoxin were different, but at the level of standardization each antitoxin neutralized approximately 30 LD_{50} of its homologous toxin.

Table 1 — Units of Porton antitoxin used for standardization of toxin.

Type	Units/ml
A	0.02
B	0.005
C	0.02
D	0.16
E	0.0125

In preliminary experiments guinea pigs immunized with univalent toxoids and challenged intraperitoneally withstood 100,000 to 1,000,000 MIP LD_{50}'s of homologous toxin; the antitoxin titers for each type at the time of challenge were approximately two times the lowest measurable titer. The lowest measurable titers were, therefore, though somewhat arbitrarily, selected as satisfactory antitoxin levels.

ASSESSMENT IN MAN

Serum antitoxin titers of individuals who had received botulinum toxoids were determined at various intervals after primary immunization and again after booster immunization. Efficacy of the toxoids is generally expressed in terms of percentages of individuals developing these or greater titers.

The first studies of man were carried out with the univalent type A toxoid containing 10.2 limit-of-flocculation dose (Lf) per milliliter. Three schedules of immunization were investigated: 0, 2, 4, and 6 weeks; 0, 2, and 4 weeks; and 0 and 8 weeks. The groups consisted of 11 to 35 persons. A single injection produced a measurable quantity of antitoxin in a few individuals. Although the 8-week injection on the 0-8 schedule appeared to have some boostering effect, the 0-2-4-6 schedule showed the highest number of persons with measurable titers at 12 weeks. Toxoids containing 1.7 and 0.34 Lf per milliliter did not appear to be as satisfactory for rapid immunization as the 10.2 Lf toxoid. Booster injections consisting of 0.25 milliliter of the 10.2 Lf toxoid were given to all individuals 5 to 7 months after the initial injection of toxoid. Satisfactory antitoxin levels were present in all persons 1 month after the booster, irrespective of the primary immunization schedule. Although the reduced Lf toxoids exhibited a high booster response, immunization with the 10.2 Lf toxoid on a 0-2-4-6-week schedule appeared to be most satisfactory for rapid immunization. Available data indicated that with the 10.2 Lf product, postbooster titers were maintained at a satisfactory level for at least 5 years.

Two purified bivalent type AB toxoids, containing 10.2 Lf of type A and 16.2 Lf of type B toxoid per milliliter were also studied in man.[58] Four schedules of immunization were investigated with groups of 25 to 50 individuals with the first bivalent preparation: 0.5 milliliter doses were given subcutaneously at 0 and 8 weeks; 0 and 10 weeks; 0, 2, and 10 weeks; and 0, 2, 4, and 6 weeks. A 0.5 milliliter booster dose of toxoid was given 1 year after the initial injection, except to individuals on

the 0-10-week schedule, to whom boosters were given after 6 months. Serum antitoxin titers of individuals were determined 2 to 3 weeks after the initial series and again 8 weeks after the booster.

The effect of schedule on the primary antitoxin response is shown in Figure 1. The striated bars represent type A response and the solid bars type B. The graph shows the percentage of persons that had measurable types A and B titers following initial immunization. Numbers in the bars are the weeks after the initial injection of toxoid.

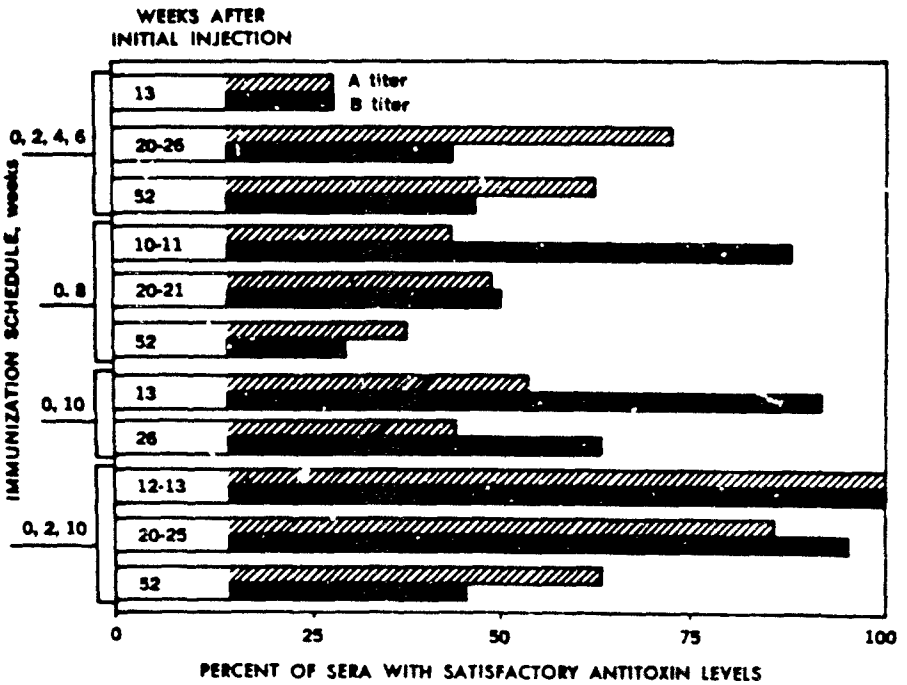

Figure 1 — Effect of schedule of immunization on antitoxin responses in man (From Fiock et al. Reference 58).

The percentage of individuals having demonstrable titers following the initial series of injections depended upon the immunization schedule. Injections administered at 0, 2, and 10 weeks produced the greatest responses, and this schedule was selected for routine immunization of man.

Marked booster responses were obtained in all cases irrespective of primary immunization schedule; a 12-month booster appeared to be more effective than a 6-month booster. Available data showed that type A and type B titers were maintained at a satisfactory level for at least 2 years after the 52-week booster. These results indicate that when a rapid, high initial response is not desired an 0-8- or 0-10-week schedule with a 52-week booster would be quite satisfactory.

Experiments were then conducted to extend observations on univalent type A and bivalent type AB toxoids to pentavalent preparations containing the five purified toxoids in combination with aluminum phosphate.[59] On the basis of exploratory investigations, two combinations of the five toxoids were formulated.

The composition of the pentavalent toxoids is shown in Table 2; the first preparation was referred to as ABCDE-1. Concentrations of type A, type B, and type D antigens are expressed in Lf. Porton antitoxins were used for the Lf measurements. The type E antigen in this toxoid was also expressed in terms of Lf, but difficulty was encountered with the flocculation of the purified type E toxins, and in toxoids 6, 7, and 8 the concentrations of type E expressed in terms of LD_{50} of the purified toxin before detoxification. The purified type C toxin did not flocculate; therefore, the concentrations of all type C antigens were expressed in terms of LD_{50} equivalents also. Five univalent preparations were also prepared, each of which contained the same concentration of antigens as in pentavalent toxoid 1 (pentavalent-1). A second pentavalent preparation, pentavalent-2, contained each toxoid at one-fifth the concentration present in the first. All toxoids contained 7 milligrams per milliliter of aluminum phosphate.

Table 2 — Antigens in pentavalent toxoids.

Type	Concentration
A	1.7 Lf per ml
B	0.54 Lf per ml
C	50,000 LD_{50} equivalents per ml
D	4.0 Lf per ml
E	5.0 Lf per ml (ABCDE-1) 100,000 LD_{50} equivalents per ml (ABCDE-6, 7, and 8)

Seventeen persons were immunized with the pentavalent-1 toxoid, and five or six persons per type were immunized with the univalent control toxoids, on a 0-2-10-week schedule. Individual titers were determined 12 and 52 weeks after the initial injection; boosters were given at 52 weeks; and titers were determined 8 weeks after the booster injection.

Data on antitoxic response to toxoid ABCDE-1 12 weeks after the initial injection and 8 weeks after the booster dose are presented in Table 3. Antigenic responses to all antigens were found 12 weeks after the initial injection. Though not shown, a considerable drop in titer occurred between 12 and 52 weeks. Fifty-two weeks after the initial injections a small percentage had measurable types A, B, C, and D titers and 94 percent had measurable type E titers. With the administration of a booster, all titers rose above the levels that had been obtained at 12 weeks. Eight weeks after the booster, one individual immunized with the pentavalent toxoid did not have a measurable type B titer; all other individuals had titers to all antigens. The median titers for each type ranged from 20 to 640 times the lowest measurable level. The univalent groups were not large enough for adequate comparison of the univalent and pentavalent responses; however, statistical analysis by the rank sum method showed that the individual responses to the pentavalent toxoid were generally not significantly different from the corresponding univalent responses at either the 12-week or the 8-week postbooster bleeding.

A major objective of our program was to obtain a high percentage of persons with measurable levels of antitoxin after the initial series of injections. Preliminary data indicated that an increase in time between the second and third injection in the initial series increased the percentage of individuals with measurable titers. Three additional pentavalent toxoids were studied simultaneously.

Table 3 — Antitoxic response of man to pentavalent toxoid ABCDE-1.

Bleeding time	Type	% with measurable titers	Porton units/ml of serum	
			Median	Range
12 weeks after first injection	A	65	0.05	<0.02 – 0.3
	B	82	0.03	<0.005 – 0.2
	C	88	0.2	<0.02 – 1.2
	D	47	<0.16	<0.16 – 1.9
	E	94	0.3	<0.0125 – 2.0
8 weeks after booster	A	100	0.6	0.03 – 6.4
	B	94	0.1	<0.005 – 1.3
	C	100	1.2	0.1 – 13.3
	D	100	4.0	0.5 – 51.2
	E	100	8.0	0.3 – 80.3

Groups of 30 persons were immunized with pentavalent toxoids 6, 7, and 8 on a 0-2-12-week schedule; a 0.5 milliliter booster was given 52 weeks after the initial injection. All individuals were bled 14 and 52 weeks after the initial injection and 8 weeks after the booster. Antitoxin titers were determined for each type on each serum.

The percentages of individuals exhibiting measurable titers 2 weeks after completion of the initial series with each of the pentavalent toxoids are presented in Table 4. Data obtained with pentavalent-1 toxoid are also presented for comparison. Two weeks after completion of the initial series a large proportion of the individuals had titers to all types regardless of the toxoid used. Response to the type C antigen was excellent in all preparations, and high percentages of individuals had type A, type B, and type E titers. The poorest response was exhibited with the type D antigen. The median titers ranged from less than measurable with the type D to as high as 24 times the measurable level with type E.

Table 4 — Comparative initial responses of man to four pentavalent toxoids 2 weeks after third injection.

		Toxoid[a] ABCDE-1	Toxoid[b] ABCDE-6	Toxoid[b] ABCDE-7	Toxoid[b] ABCDE-8
Type A	% Measurable	65	90	97	61
	Median, units/ml	0.05	0.2	0.2	0.08
Type B	% Measurable	82	93	80	59
	Median, units/ml	0.03	0.03	0.02	0.008
Type C	% Measurable	88	100	87	89
	Median, units/ml	0.2	0.3	0.1	0.1
Type D	% Measurable	47	79	60	52
	Median, units/ml	<0.16	0.5	0.5	0.2
Type E	% Measurable	94	100	90	63
	Median, units/ml	0.3	0.2	0.08	0.02

[a]Toxoid 1, 0-2-10-week schedule.
[b]Toxoids 6, 7, and 8, 0-2-12-week schedule.

The general trend appeared to be toward an increased response with the 0-2-12-week schedule, and this was selected for routine immunization. All titers declined between 14 and 52 weeks and only a small portion of the individuals had measurable titers at 52 weeks.

The data obtained 8 weeks after the 52-week booster are shown in Table 5; a booster given at 52 weeks was very effective, and with toxoids 6, 7, and 8, all except three titers were measurable 8 weeks after the booster. The median titers and percentages with measurable titers are summarized here. In all cases the median titers were less than the mean titers; means ranged from 1.3 to 5.5 times the median titers. After the initial series, median titers were 2 to 16 times the lowest measurable level; after the booster, they ranged from 10 to 100 times the measurable level. Responses after the booster were similar to those obtained with toxoid 1.

Table 5 — Comparative responses of man to four pentavalent toxoids 8 weeks after booster.

		ABCDE-1 Toxoid	ABCDE-6 Toxoid	ABCDE-7 Toxoid	ABCDE-8 Toxoid
Type A	% Measurable	100	100	100	100
	Median, units/ml	0.6	2.1	2.1	0.8
Type B	% Measurable	94	100	86	94
	Median, units/ml	0.1	0.3	0.2	0.03
Type C	% Measurable	100	100	100	100
	Median, units/ml	1.2	1.2	0.8	0.6
Type D	% Measurable	100	100	89	100
	Median, units/ml	4.8	2.4	1.2	2.0
Type E	% Measurable	100	100	100	100
	Median, units/ml	8.0	1.0	0.2	0.2

EVALUATION IN ANIMALS

Throughout the development and evaluation studies, attention was given to the response of laboratory animals to botulinum toxoids. In animals, estimation of the antigenicity of botulinum antigens is based not only on development of serum antitoxin titers, but also on development of resistance to challenge with toxin. Titrations carried out in mice[55, 56, 57, 60, 61] showed the adsorbed toxoids to be highly antigenic. Maximum protection to intraperitoneal challenge was obtained 3 to 5 weeks after immunization. At this time the immunized animals survived 10^3 to 10^6 mouse LD_{50}. Addition of the aluminum phosphate adjuvant markedly increased the antigenicity of the toxoids for mice. With the type C toxoid, protective activity of the adsorbed preparations was approximately 30 times as great as that obtained with the non-adsorbed.

Early in our studies consideration was given to the relationship between the serum antitoxin titer and the degree of resistance to botulinum intoxication. Experiments were carried out with guinea pigs. Guinea pigs were immunized subcutaneously with aluminum-phosphate-adsorbed univalent toxoids and challenged intraperitoneally with homologous toxin. Serum samples were obtained the day before challenges for antitoxin determinations. Figure 2 shows the resistance to intraperitoneal challenge as a function of antitoxin titer in guinea pigs. The log of the average antitoxin titers is shown on the horizontal axis. Arrows on this axis designate

the lowest measurable titer for each type antitoxin. The 50 percent survival end point, expressed in MIP LD_{50} is shown on the vertical axis. As antitoxin titer increased, the level of resistance also increased and in several cases appeared to reach a maximum.

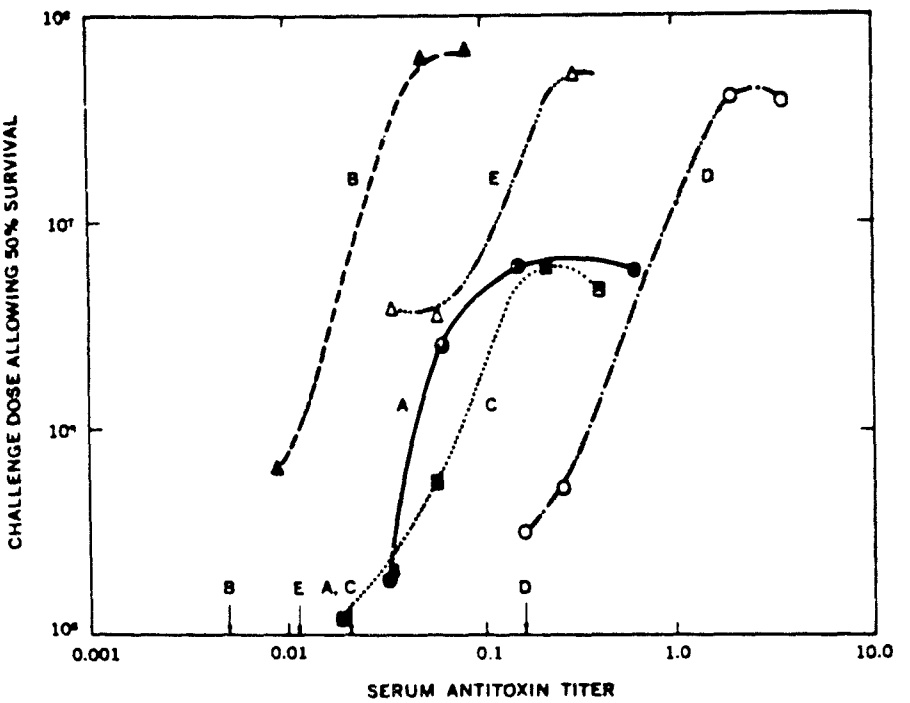

Figure 2 — Resistance to intraperitoneal challenge as a function of serum antitoxin titer in guinea pigs.

Additional studies were carried out with rabbits and guinea pigs to determine the antigenic response to univalent and pentavalent toxoids as a function of type, dose, time, and a booster dose of toxoid.[62]

Separate groups of rabbits and guinea pigs were given subcutaneous injections of the pentavalent-1, pentavalent-2, and the five univalent control toxoids described earlier. A booster dose was given to rabbits after 90 days and to guinea pigs after 180 days. Antitoxin titers were determined at intervals after immunization.

A comparison of responses of the type B antitoxin in rabbits to the univalent and pentavalent toxoids is shown in Figure 3 for the purpose of illustrating the nature of the responses. The maximum response to the type B antigen in rabbits after a single injection of univalent or pentavalent toxoids was obtained in approximately 50 days. In no case did the response to the B antigen in the pentavalent preparations reach the response to the univalent antigen. Response to the B antigen in the pentavalent-2 toxoid was also lower than the response to this antigen in the pentavalent-1 toxoid. Titers increased following the 90-day booster injections. Responses paralleled each other, both before and after the booster injections, and the best booster response was obtained with the pentavalent-2 toxoid. The results obtained in guinea pigs were generally similar. Though not shown, all toxoid components given

individually or in combination stimulated satisfactory antitoxin responses following primary immunization, and the titers increased following booster injections. Antibody formation was more rapid with the more concentrated pentavalent toxoid in both rabbits and guinea pigs. The results with both species are summarized in Table 6.

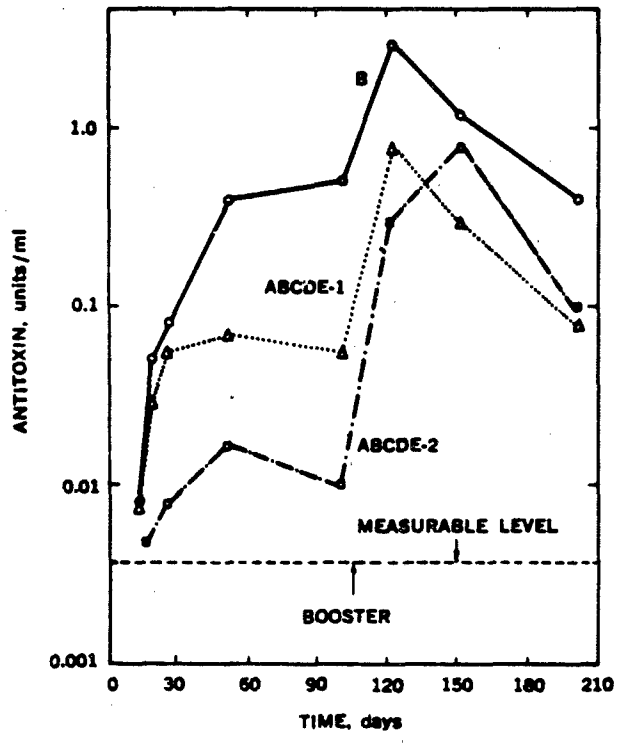

Figure 3 — Type B antitoxin response in rabbits.

Table 6 — Maximum initial antitoxin responses to ABCDE-1 relative to univalent controls (titer with pentavalent/titer with univalent expressed in percent).

Type	Rabbits	Guinea pigs
A	10	140
B	14	4
C	95	20
D	33	14
E	37	20

With few exceptions, the results indicate that formation of individual antibodies was reduced when the toxoids were given in a pentavalent combination. The repression appeared to be less marked during the secondary response following booster. In general, a slower rate of antibody formation and a more marked repression were observed in guinea pigs than in rabbits. Data are not available for conversion of the antitoxin units to weight units, and accordingly, the total amount of antibody produced in response to the pentavalent combination cannot be compared in absolute terms with the univalent controls. It would appear, however, that the total antibody

response to the combination was not greatly different from the response to the univalent antigen. It seems probable that the decreased individual responses represented a competition of the antigens for the antibody-forming mechanism, or possibly for the adjuvant activity of the aluminum phosphate.

The immunizing efficacy of botulinum antigens was evaluated further by administering toxin to immunized animals by various routes to determine their resistance. It has been established that inhalation of botulinum toxins in moderate doses is lethal to laboratory animals.[66] It seemed possible that respiratory challenge might provide a more severe test of the efficacy of toxoids than challenge by other routes. Support for this view was provided by Iakolev's finding that higher levels of antitoxin were required for prophylaxis against inhalation of botulinum toxin than against toxin administered by other routes.[67, 68] The results of some of our studies to determine resistance afforded actively immunized guinea pigs are presented below.[63]

The toxins used in our initial studies were produced from cultures grown in cellophane tubing immersed in nutrient medium. Cultures were clarified by centrifugation and used without further concentration or purification, since unaltered cell-free preparations were considered to be most suitable. Type E toxin was activated with trypsin prior to clarification of culture and subsequently stored in the frozen state.

Intraperitoneal and oral LD_{50} estimates of toxins were determined for mice and guinea pigs. Respiratory LD_{50} estimates were determined for guinea pigs exposed bodily to static aerosols of the toxins. Over 60 percent of the particles in the aerosols were 5 microns or less in diameter. The guinea-pig-inhaled dose, in MIP LD_{50} units, was estimated by titration of the collecting fluid from impinger samplers. Serum antitoxin titers of the immunized animals were determined on sera obtained by cardiac bleeding of the animals the day before challenge.

Before immunized guinea pigs were challenged, the toxicities for normal guinea pigs were determined. Estimates for the five toxins administered by the various routes are summarized in Table 7. The results are expressed in MIP LD_{50}. The animals were most susceptible by the intraperitoneal route, as expected, and were more susceptible to toxin administered by the respiratory route than by the oral route.

Table 7 — Toxicity of botulinum toxins administered to normal guinea pigs by various routes.[a]

Type	Intraperitoneal	Respiratory	Oral
A	5.2	141	717
B	4.2	350	306
C	1.6	87	177
D	4.1	186	436
E	34.3	778	178,000

[a]Results expressed in MIP LD_{50}.

Expression of the respiratory LD_{50} estimates in terms of guinea pig intraperitoneal LD_{50} revealed that guinea pigs show essentially similar susceptibility to the five toxins administered by the respiratory route. The respiratory susceptibility is more closely related to the oral susceptibility than to the intraperitoneal susceptibility with four of the five types.

In our first series of experiments, groups of guinea pigs were immunized by the subcutaneous route, each with a single injection of univalent toxoid. Each of the

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univalently immunized groups of guinea pigs was divided into three subgroups, which were challenged 36 to 40 days after immunization with homologous toxin by the oral, respiratory, and intraperitoneal routes, respectively. Normal control groups were challenged in the same manner.

The respiratory challenge results are summarized in Table 8. It may be noted that 79 to 91 percent of the actively immunized guinea pigs survived this level of respiratory challenge. Although not shown in this table, 70 to 100 percent of the subgroups challenged by the intraperitoneal and oral routes, respectively, survived challenge of 10^4 to 10^8 LD_{50}.

Table 8 — Responses of immunized guinea pigs to respiratory challenge with botulinum toxins.

Type	Antitoxin titer[a]	Challenge, LD_{50}[b]	No. survivors / Total exposed	Percent survival
A	0.3 <0.04 – 0.96	5	40/44	91
B	0.059 <0.005 – 0.8	7	30/38	79
C	0.4 <0.02 – 1.9	8	38/44	86
D	6.1 <0.32 – 21	9	35/39	90
E	0.29 <0.025 – 2.0	3	26/32	81

[a]Average and range.

[b]Guinea pig respiratory LD_{50}.

Average antitoxin titers ranged from 10 to 50 times the minimal measurable levels. Although a considerable variation in antitoxin titers was obtained, the high percentage of survivors made it impractical to test for a relationship between serum antitoxin titers of individual animals and resistances to challenge. It appeared evident from these results that higher levels of challenge would be necessary to determine the magnitude of resistance to respiratory challenge. A second test in this series of experiments was designed to accomplish this.

Groups of guinea pigs were immunized with either univalent type D or pentavalent botulinum toxoids and subsequently challenged with graded doses of toxin by the respiratory route. Type D was selected because the toxicity of the culture permitted higher challenge doses. Immunization with pentavalent toxoid was included so that effectiveness of an antigen in a multivalent preparation and a univalent antigen could be compared. Results of this test are shown in Table 9.

A half-milliliter immunizing dose was employed for the univalent group and for the first pentavalent group, and these groups were challenged 40 days after immunization. The second pentalavent group received 1.0 milliliter as an immunizing dose and was challenged 50 days after immunization. Subgroups were challenged with graded doses of type D toxin by the respiratory route. Smaller groups of guinea pigs immunized in the same manner were challenged by the intraperitoneal and oral routes.

Table 9 — Responses of immunized guinea pigs to respiratory challenge with type D toxin.

Type immunization	Antitoxin titer[a]	Challenge, LD_{50}[b]	No. survivors / Total exposed	Percent survival
Univalent (0.5 ml)	3.8	22	40/40	100
		162	40/40	100
	3.2 – 5.1	2,100	40/40	100
Pentavalent (0.5 ml)	0.63	26	20/20	100
		277	20/20	100
	<0.16 – 1.9	2,090	16/20	80
Pentavalent (1.0 ml)	1.5	26	20/20	100
		230	20/20	100
	0.32 – 5.8	1,980	20/20	100
None	————	1	6/20	30

[a]Average and range of serum pools obtained day before challenge.
[b]Guinea pig respiratory LD_{50}.

Essentially all of the actively immunized guinea pigs survived 20 to 2,000 respiratory LD_{50}. Although not shown here, 60 to 100 percent of comparable immunization groups survived 10^6 guinea pig LD_{50} when challenged either by the intraperitoneal or by the oral route.

Additional experiments were conducted with dynamic aerosols generated in the Henderson apparatus. Groups of guinea pigs were immunized with a 0.5-milliliter dose of pentavalent toxoid and challenged after 40 days with a single type toxin by the intraperitoneal, oral, and respiratory routes.

Table 10 summarizes results obtained with types A and D challenges. Immunization of guinea pigs with pentavalent toxoid afforded a high level of protection against challenge by the three challenge routes. Resistance to challenge by the respiratory route was similar to resistance to challenge by the intraperitoneal and oral routes. Essentially similar results were obtained with the types B, C, and E toxins.

Table 10 — Resistance of pentavalently immunized guinea pigs to A and D toxins administered by various routes.

Type of toxin	Route of challenge	Challenge, LD_{50}[a]	No. survivors / Total exposed	Percent survival
A	Intraperitoneal	3×10^6	8/15	53
	Oral	5×10^5	15/16	94
	Respiratory	3×10^4	11/12	92
D	Intraperitoneal	2×10^6	8/14	57
	Oral	4×10^4	8/10	80
	Respiratory	2×10^4	6/13	46

[a]Guinea pig LD_{50}.

SUMMARY

Procedures have been developed for preparation of purified, aluminum-phosphate-adsorbed, univalent, bivalent, and pentavalent botulinum toxoids for the immunization of man. All preparations were well tolerated and elicited satisfactory antitoxin responses in man, whether administered as a single antigen or in combination. Four separate pentavalent type ABCDE toxoids produced immune responses to each antigen in a considerable proportion of individuals following an initial series of three injections. A booster injection administered 1 year after the initial injection markedly increased the antitoxin titers, and measurable antitoxin titers were found in 86 to 100 percent of the individuals immunized. The toxoids were antigenic for mice, guinea pigs, and rabbits. In guinea pigs, the toxoids afforded a high level of resistance to challenge by toxins administered by various routes.

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DISCUSSION FROM THE FLOOR

Dr. Murray: This paper certainly has indicated the possibilities of active immunization, and much of the data presented, at least in the case of animals, would indicate the relationship of the antibody levels that must be present to protect against the different amounts of toxin given by different routes. I think this, in itself, is important information.

Dr. Rogers, Nashville: Mr. Cardella, I am very impressed with the total response. Did you do any studies on the incidence of antibody response after the first, second, and third injections of toxoid?

Mr. Cardella: With the pentavalent immunizations, we did not. In a small group of individuals, however, antitoxin titers were determined following immunization with the univalent and the bivalent antigens. Indications were that a very small percentage developed antitoxin titer after a single injection of a more concentrated toxoid. A higher percentage had titers after the second and, of course, as described, after the third injection, or after a fourth. A higher proportion had measurable titers after the series and this, as I described, was the basis for spacing the injections to take advantage of what appeared to be anamnestic reactions.

From the audience: How did you establish the dose that you applied in the inhalation challenge?

Mr. Cardella: We first determined the breathing rates and volumes of the animals exposed to the aerosols. During animal exposure, the concentration of toxin in the aerosols was determined by obtaining air samples in appropriate pre-rated air samplers containing collecting fluid. This fluid was titrated for toxin concentration. Estimates of animal dose inhaled were made from the data obtained, based upon the relationship between concentration of toxin per volume of air sampled and volume of air inhaled by the animals.

From the audience: Could you say something about possible reactions to the toxoid?

Mr. Cardella: We have seen what we consider to be mild to moderate local reactions. I think some of you may have observed this. It has been described as a small nodule of varying size that persists for varying periods of time. Incidence of reactions with the pentavalent toxoid is about 1 percent. Approximately 1,000 individuals have received this toxoid. Indications are that the percentage of reactions to the pentavalent may be slightly higher than to our univalent or bivalent preparations. This may be due to the adjuvant, which has been modified slightly. We are now using a Holt's ⅔ aluminum phosphate gel that is ⅔ aluminum phosphate and ⅓ aluminum hydroxide. The previous gel was essentially all phosphate. In our pentavalent preparations, the types C, D, and E antigens were not taken to the degree of purity that the A and B were. It is possible that inclusion of one of these preparations might account for the slightly higher reaction rate; the rate is relatively low, however.

Dr. Petty, Baltimore: Did you see any total failures of response of humans to the pentavalent toxoid? If so, can these be explained in any manner, and would you consider these people resistant to botulinum toxin?

Mr. Cardella: We saw lack of response to any antigen in the primary immunizations. We have followed approximately 120 individuals, four of whom showed no response to any of the five antigens after the initial series. I might point out that approximately 41 percent showed titer to all five antigens and at least 79 percent showed measurable titer to at least four of the five antigens. The incidence of no measurable response was fairly low.

Failure to demonstrate antitoxin titers following toxoid injections could be attributed to the limit of sensitivity of the neutralization test employed to measure titers. With regard to considering these people resistant to botulinum toxin, it can be pointed out that laboratory animals with no demonstrable antitoxin titers following an injection of toxoid were resistant to toxin. Actually, the apparent non-responder may have produced antitoxin that is not measurable by our test. For the absolute non-responder I would not recommend a willful exposure to toxin.